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Chemokine profiles in blood associated with delayed asthmatic response to allergen challenge

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KEYWORDS
Allergic bronchial asthma;
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Summary
Background: Patients with allergic bronchial asthma having been challenged with allergen develop various types of asthmatic response, such as immediate (IAR), late (LAR) or delayed (DYAR) response, due to different immunologic mechanisms. The DYAR, beginning 26–32 h, reaching maximum between 32 and 48 h and resolving within 56 h after the challenge, differs from IAR and LAR in clinical and immunologic features.
Objectives: To investigate the changes in the serum concentrations of chemokines associated with the isolated form of DYAR.
Methods: In 22 patients the repeated DYAR ($p < 0.001$) was supplemented with recording of blood cell counts and serum concentrations of chemokines before, and up to 72 h after the bronchial challenge by means of enzyme-linked immunoassay, (ELISA).
Results: The DYAR was associated with (a) significantly increased serum concentrations ($p <$ [illegible]) of CCL 2, CCL 3, CCL 4, CCL 7, CCL 20, CXCL 1, CXCL 8, CXCL 9, CXCL 10 and CXCL 11, and (b) significantly decreased serum concentrations, ($p < 0.05$) of CCL 5, CCL 11, CCL 17, CCL 22, CCL 24 and CCL 26, as compared with their pre-challenge as well as the PBS control values. No significant chemokine changes were recorded during the PBS controls ($p > 0.1$).
Conclusions: These results, together with changes in the blood cell counts, provide evidence for an involvement of activated Th_1, cells and NK cells (CCL-2, -3, -4, -20, CXCL-9,-10,-11), neutrophils (CCL-20, CXCL-1,-8) and monocytes (CCL-2,-3,-4, -7, CXCL-10), upon co-operation of other cell types, such as epithelial, endothelial and dendritic cells, in the immunologic mechanism(s) underlying the DYAR.

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Introduction

Various immunologic mechanisms can be involved in various clinical phenotypes of allergic bronchial asthma.[1–6] Besides the already established classic immediate hypersensitivity based upon participation of IgE antibodies, mast cells/basophils, eosinophils and Th_2-lymphocytes, other hypersensitivity mechanisms can also play a role in this condition.[1–15] Patients with bronchial asthma develop various types of asthmatic response to bronchial allergen challenge, such as immediate (IAR),late (LAR) or a dual late (DLAR; a combination of an immediate and a late) asthmatic response. The IAR, LAR and DLAR have already been extensively studied from various points of view.[5,12,16–28]

Some patients with bronchial asthma, examined at our clinic, developed an asthmatic response appearing 26—56 h after the bronchial challenge with various inhalant allergens.[29–32] This response phenotype, designated by us as "delayed asthmatic response" (DYAR), displayed clinical and immunologic features different from those associated with the IAR and the LAR.[2,3,5–12,19,21,25–28]

The key clinical aspect of the DYAR was it onset later than 26 h after the allergen exposure,[29–32] whereas the most important immunologic feature seems the predominant role of activated Th_1 —cells, neutrophils and monocytes in the mechanism(s) underlying the DYAR.[29–32] The DYAR was associated with increased counts of total leukocytes, lymphocytes, neutrophils and shifting of the Th_1/Th ratio in favor of Th_1 cells in peripheral blood,[29–32] increased intracellular concentrations of IFN-γ and IL-2, but not of IL-4 or IL-5, in PBMC,[29] increased serum concentrations of sICAM-1, sVCAM-1, sPECAM-1, sE- and sL-selectins, whereas decreased concentration of sE-cadherin,[30] increased plasma concentrations of LTB_4 and MPO,[31] and increased serum concentrations of some cytokines, such as IFN-γ, IL-2, IL-18, G-CSF, TNF-α and TGF-β.[32]

The purpose of this study was to search for the possible changes in concentrations of other important components of immunologic system, the particular chemokines, in peripheral blood during the DYAR and in this way to contribute not only to assessment of the role of individual cell types in the DYAR, but also to the clarification of the immunologic mechanism(s) underlying this response type.

Methods

Patients

Some of bronchial asthma patients referred to our Department of Allergology &Immunology, Inst. Med. Sci. *De Klokkenberg*, Breda, The Netherlands, for more extensive diagnostic and therapeutic analysis have developed an isolated form of delayed asthmatic response (DYAR), appearing later than 24 h after the bronchial challenge with allergen (Table 1, Fig. 1). Twenty-two patients developing the DYAR have volunteered to participate in this study (Table 1).

These patients, 19—43 years of age, suffering from reversible bronchial constriction, alternating with symptom-free periods, showed pulmonary function without any restrictive changes (GINA).[33] They did not suffer from current airway infections and did not use oral corticosteroids or receive immunotherapy. They were examined by routine diagnostic procedure, serving also as an exclusion criteria, consisting of a number of diagnostic parameters

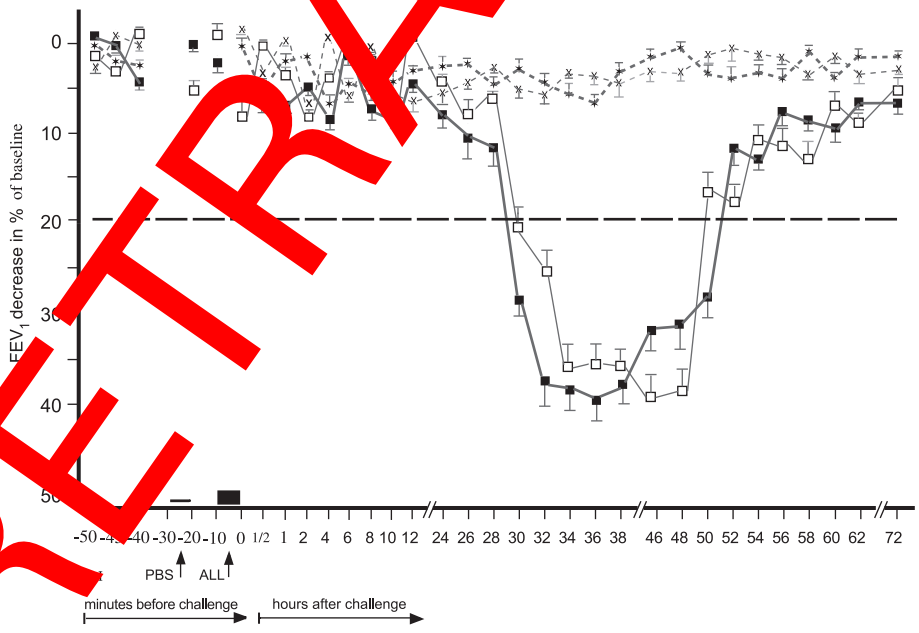

Figure 1 The initial and repeated delayed asthmatic response to allergen challenge (DYAR) and phosphate buffered saline (PBS) control challenge. The mean percentage changes in the FEV_1 values with respect to the baseline values were calculated from 22 DYARs and 22 PBS control challenges; (□) = initial DYAR; (■) = repeated DYAR; (*) = initial PBS; (×) = repeated PBS. I = initial (baseline) values; ALL = allergen challenge; PBS = phosphate buffered saline; Bars = means ± SEM; Dashed horizontal line = statistically significant decrease of FEV_1 values (>20%).

and including also 53 BPTs with allergens and 22 PBS control challenges (Tables 1 and 2). All BPTs were performed under standard and controlled conditions in a period without manifest bronchial complaints and during hospitalization of the patients.

Inhalation glucocorticosteroids ($n = 11$) and long-acting β_2-sympathomimetics ($n = 9$) were withdrawn 4 weeks prior to BPTs, disodium cromoglycate ($n = 5$), nedocromil sodium ($n = 6$) 2 weeks before challenges and other drugs 48 h prior to BPTs. If the post-challenge FEV_1 or both FEV_1 and FVC values decreased by 50% or more, with respect to the predicted values, the patients ($n = 3$) were treated with a single dose of 400 mcg aerosolized salbutamol.

The supplementary parameters and the chemokine array were determined in all patients studied and in control subjects as a single test during the routine procedure (Tables 1 and 3).

The BPTs with the inhalant allergens producing the DYAR ($n = 22$) (Table 2) and the PBS control challenges ($n = 22$) were repeated 3–4 weeks later (Fig. 1) and supplemented with recording of cell counts and chemokines in the peripheral blood before, and 1, 12, 24, 36, 48, 56 and 72 h after the challenge (Tables 4 and 5). The local ethics committee (IRB-MCK) approved this study and a written informed consent has been obtained from all participants. The study has been carried out according to the WMA Declaration of Helsinki concerning the Principles for medical research involving the human subjects.

Control subjects

Thirty-five control subjects volunteered to participate in this study; 9 asthmatics demonstrating an isolated IAR to allergen challenge, 8 asthmatics developing an isolated LAR, 6 asthmatics showing DLAR and 12 healthy subjects (Table 1).

Allergens

Dialyzed and lyophilized allergen extracts (Allergopharma, Reinbek, Germany) diluted in PBS were used in

Table 1 Characteristics of the patients and control subjects.

	Patients DYAR $n = 22$	Control subjects			
		Patients with asthma			Healthy subjects $n = 12$
		IAR $n = 9$	LAR $n = 8$	DLAR $n = 6$	
Age (years)	33 ± 5	33 ± 3	31 ± 2	29 ± 3	32 ± 6
Gender (M/F)	10/12	5/4	4/4	2/4	5/7
Disease history (years)	4.3 ± 2.5	4.8 ± 2.4	5.1 ± 1.6	5.0 ± 1.9	0
Asthmatic attacks per month	2 ± 1	2 ± 1	3 ± 1	3 ± 2	0
FEV_1 (% predicted)	94.5 ± 7.2	95.3 ± 5.1	90.9 ± 5.4	92.7 ± 4.5	98.8 ± 3.1
FVC (% predicted)	97.6 ± 6.4	98.3 ± 5.9	99.4 ± 4.7	101.1 ± 3.9	103.2 ± 6.0
Blood leukocyte count (x 10^9/L)[a]	11.2 ± 0.6	6.9 ± 0.7	8.1 ± 1.2	8.8 ± 0.9	6.3 ± 0.8
Blood neutrophil count (x 10^9/L)[b]	7.6 ± 1.6	4.7 ± 1.3	5.9 ± 2.4	6.3 ± 1.5	6.1 ± 2.3
Blood eosinophil count (x 10^6/L)[c]	314 ± 27	**463 ± 56***	**492 ± 41***	**502 ± 54***	287 ± 29
Skin prick tests response					
-immediate	7	9	4	2	0
Intracutaneous tests response					
-immediate	6	8	5	4	0
-late	3	1	3	2	0
-delayed	10	0	0	0	0
Bronchial histamine threshold[d]					
≤2.0 mg/mL	0	1	0	1	0
4.0 mg/mL	3	3	1	1	0
8.0 mg/ml	2	1	4	3	0
16.0 mg/mL	10	2	1	1	0
32.0 mg/mL	3	2	1	0	1
>32.0 mg/mL	5	0	1	0	11
Ratio Th_1/Th_2 (%) in blood[e]	6.2 ± 2.5	7.3 ± 2.8	7.5 ± 2.2	7.7 ± 3.3	7.6 ± 1.9
IFN-γ (pg/mL)—PBMC[f]	194 ± 61	155 ± 48	167 ± 55	171 ± 46	188 ± 52
IL-4 (pg/mL)[f]	18.0 ± 5.4	22.3 ± 6.5	20.9 ± 3.7	21.0 ± 4.2	19.5 ± 4.7

DYAR = delayed asthmatic response; IAR = immediate asthmatic response; LAR = late asthmatic response; DLAR = dual late asthmatic response (combination of immediate and a late response); Values = mean ± SD; Statistical significance as compared with healthy control subjects: $^*p < 0.05$ (in bold).

[a] Normal value = 4.0–10 × 10^9/L.

[b] Normal value = 2–7.2 × 10^9/L.

[c] Normal value = <300 × 10^6/L.

[d] Normal value >32.0 mg/mL.

[e] Determined by means of flow-cytometry after stimulation with PMA (phorbol 12-myristate 13-acetate) (mean ± SEM).

[f] Determined by means of ELISA after "in vitro" stimulation of peripheral blood mononuclear cells (PBMC) with PMA (mean ± SEM).

Table 2 Allergen inducing particular types of asthmatic response.

Allergen	Standard concentrations BU/mL (i.c./BPT)	DYAR						NAR					
		Positive skin response (n = 22)				DYAR (n = 22)		Positive skin response (n = 31)				NAR (n = 31)	
		ISR	LSR	DYSR	Total			ISR	LSR	DYSR	Total		
		1:1	1:5/1:1	1:5/1:1		1:10/1:1	Total	1:5/1:1	1:5/1:1	1:5/1:1		1:10/1:1	Total
Dermatophagoides pteronyssinus	100/1000	1/1	0/1	1/1	4	1/3	4	1/1	0/1	0/1	3	0/0	3
Dermatophagoides farinae	100/1000	0/0	1/0	0/0	1	1/—	1	0/0	0/1	0/1	2	0/0	2
Animal danders													
-dog	300/3000	0/0	0/0	0/1	1	0/1	1	0/1	0/0	0/1	2	0/0	2
-cat	100/1000	0/1	0/1	1/1	2	1/1	2	1/1	0/1	0/1	4	0/0	4
-horse	200/2000	0/0	0/0	0/1	1	0/1	1						
-hamster	200/2000	1/0	0/0	0/0	1	0/1	1						
Aspergillus fumigatus	100/1000	0/0	0/1	0/0	1	0/1	1						
Pollen													
-grass mix I	100/1000	1/1	0/0	1/2	5	1/4	5	1/2	0/1	0/3	7	0/0	7
-grass mix II	100/1000	0/1	0/0	0/0	1	0/1	1	0/1	0/1	0/0	2	0/0	2
-tree mix	300/3000	0/1	0/0	0/0	1	0/1	1	0/1	0/0	0/1	3	0/0	3
-weed mix	100/1000	0/0	0/0	0/1	1	0/1	1	0/1	0/1	0/0	2	0/0	2
-birch	100/1000	0/1	0/0	0/0	1	0/1	1	0/1	0/1	0/2	5	0/0	5
-poplar	200/2000	0/1	0/0	0/0	1	0/1	1	0/0	0/0	0/1	1	0/0	1
-ragweed giant	100/1000	0/0	0/0	0/1	1	0/1	1						

BU/mL = biologic units per 1 mL; i.c. = intracutaneous tests; BPT = bronchial provocation tests; Dilutions of allergen extract 1:5 with respect to the standard concentration; 1:1 (undiluted standard concentration); — = not performed; DYAR = delayed asthmatic response; NAR = negative asthmatic response; ISR = immediate skin response; LSR = late skin response; DYSR = delayed skin response.
Grass pollen mix I = *Dactylis glomerata*, *Lolium perenne*, *Phleum pratensis*, *Poa pratensis*.
Grass pollen mix II = *Festuca pratensis*, *Holcus lanatus*, *Agrostis alba*, *Anthoxanthum odoratum*.
Tree pollen mix = *Betula pendula*, *Corylus avellana*, *Juniperus communis*, *Salix alba*.
Weed pollen mix = *Artemisia vulgaris*, *Plantago lanceolata*, *Rumex acetosa*, Taraxacum officinale.

Table 3 Pre-challenge chemokine concentrations in the (non-stimulated) serum (pg/mL).

	Patients DYAR $n = 22$	Control subjects			
		Asthma patients			Healthy subjects
		IAR $n = 9$	LAR $n = 8$	DLAR $n = 5$	$n = 12$
CCL 1 (I-309)	<7.8	<7.8	8.9 ± 0.5	<7.8	<7.8
CCL 2 (MCP-1)	38.7 ± 1.5	41.6 ± 2.5	44.8 ± 1.9	43.4 ± 2.0	37.5 ± 1.3
CCL 3 (MIP-1α)	7.4 ± 0.2	8.3 ± 0.7	7.9 ± 0.5	9.0 ± 1.4	8.1 ± 0.6
CCL 4 (MIP-1β)	5.8 ± 1.0	9.5 ± 2.0	8.4 ± 1.1	7.5 ± 0.8	6.9 ± 2.1
CCL 5 (RANTES)	8.5 ± 2.3	10.3 ± 2.6	6.6 ± 1.7	8.0 ± 0.5	7.0 ± 1.9
CCL 7 (MCP-3)	9.0 ± 0.5	12.9 ± 3.1	14.7 ± 2.2*	14.1 ± 1.6	10.5 ± 2.0
CCL 11 (Eotaxin-1)	8.9 ± 1.7	16.7 ± 1.4*	15.9 ± 1.8	16.3 ± 1.0*	8.4 ± 0.5
CCL 13 (MCP-4)	96.3 ± 16	123.5 ± 16	138.1 ± 19*	[illegible] 8.6	73.2 ± 5.1
CCL 17 (TARC)	65.2 ± 4.9	89.3 ± 6.7*	70.8 ± 4.4	91.3 ± [illegible]	54.5 ± 3.2
CCL 18 (PARC)	25.0 ± 3.4	33.0 ± 1.6	41.2 ± 2.9	40.6 ± 1.1	31.3 ± 2.4
CCL 19 (MIP-3β)	11.5 ± 2.9	9.5 ± 1.3	14.8 ± 2.4	14.3 ± 1.0	10.5 ± 0.7
CCL 20 (MIP-3α)	18.7 ± 3.3*	3.4 ± 0.5	7.9 ± 1.1	[illegible] ± 2.5	9.3 ± 3.2
CCL 22 (MDC)	224.3 ± 36	279.0 ± 22	325.4 ± [illegible]*	31[illegible] ± [illegible]	219.0 ± 8.8
CCL 23 (MIP-3)	78.2 ± 5.1	69.2 ± 5.7	73.4 [illegible] 3.5	84.2 ± [illegible]	61.0 ± 1.4
CCL 24 (Eotaxin-2)	129.8 ± 14.	177.1 ± 12*	154 [illegible] ± 15	168.9 ± 13*	109.2 ± 4.8
CCL 25 (TECK)	<62.5 *	84.7 ± 6.3	95.1 [illegible] 7	101 [illegible] ± 9.4	85.9 ± 2.6
CCL 26 (Eotaxin-3)	173.4 ± 13	256.3 ± 27*	[illegible] 1.0 ± [illegible]	2[illegible].6 ± 31*	154.2 ± 10
CCL 27 (CTACK)	335.2 ± 29	311.0 ± 36	339.5 ± 29	24.7 ± 25	322.1 ± 18
CXCL 1 (GRO-α)	61.8 ± 9.4*	39.4 ± 3.0	42.7 ± 2.1	43.0 ± 3.3	35.1 ± 1.6
CXCL 8 (IL-8)	9.1 ± 2.0	7.4 ± 1.0	[illegible] ± 1.5*	10.5 ± 1.1	5.9 ± 0.4
CXCL 9 (MIG)	25.0 ± 4.3 *	16.8 ± 2.3	16.2 [illegible] 7	17.5 ± 1.0	14.9 ± 0.8
CXCL 10 (IP-10)	14.8 ± 3.1	11.3 ± 0	10.8 ± 1.[illegible]	10.0 ± 0.6	9.4 ± 0.5
CXCL 11 (I-TAC)	54.6 ± 5.7*	43.5 [illegible] 6	39.8 ± 1.5	41.2 ± 0.7	34.3 ± 1.1

Values = mean ± SEM; Statistical significance as compared w[illegible] healthy subje[illegible] values: *$p = < 0.05$.

concentrations of 500 BU/mL for skin prick tests (SPT), 20-300 BU/mL for intracutaneous tests (i.c.) and 100—3000 BU/mL for BPTs (Table 2). Concentrations recommen[illegible] manufacturer were up to 500 BU/mL for the skin te[illegible] and up to 5000 BU/mL for the BPTs.

Skin tests

The SPTs have been performed in co[illegible]entrati[illegible] BU/mL and evaluated after 20 min. The [illegible]ving i.c. tests were carried out in concentrat[illegible]ns of 2[illegible]0 BU/mL and 100—300 BU/mL and evaluate[illegible]20 min and t[illegible]6, 12, 24, 36, 48, 72, and if necessary [illegible]urs after the intradermal injection (Table 2). PBS w[illegible] used [illegible] negative and histamine diphosphate (0.1 and 0.01 mg[illegible]) as a positive control. Generally, th[illegible] skin "wheal and [illegible]are" reactions were always evaluat[illegible]. A skin wheal reaction (>7.0 mm in diameter) occurri[illegible]20 min [illegible]ter the intradermal injection was qualified as p[illegible]ive [illegible]mmedia[illegible] skin response, skin infiltration appearing b[illegible]ween 6 [illegible]d 12 h as a late skin response and [illegible]duratio[illegible]rded later than 48 h was considered [illegible]delay[illegible]d skin resp[illegible]nse.[16,17,29—32]

Bronchial [illegible]cation tests (BPT)

The BPTs were pe[illegible]med by means of spirometry (Spirograph D-75 Lode, Groningen, The Netherlands) recording the FVC and FEV_1 values. The aerosolized allergen extracts and PBS were inhaled using Wiebadener Doppel-Inhalator at an airflow of 10 L/min. The nebulizer output was [illegible].12—0[illegible]4 mL/min and the aerosol particles were of a [illegible]dian mass diameter of 2.8—3.6 μ. The BPTs, being a modification of the European standard technique,[34] were [illegible]formed by means of serial dilutions (Table 2) according to the following schedule: (1) initial (baseline) values recorded at 0, 5 and 10 min; (2) PBS control values recorded at 0, 5 and 10 min after a 10-min PBS inhalation; (3) inhalation of allergen aerosol for 2 × 5 min, with inserted spirometry measurement, followed by the recording of the FEV_1 and FVC values at 0, 5, 10, 20, 30, 45, 60, 90 and 120 min and then every hour up to 12th hour, every second hour during the 22nd to 38th, the 46th to 62nd and at the72nd hour interval.[16,17,29—32] The PBS control challenge was performed by the same schedule as that of the BPTs with allergens.

Supplementary parameters

The IFN-γ/IL-4 ratio (%)values in peripheral blood, indicating the Th_1/Th_2 ratio, were determined by means of flow-cytometry. The intracellular IFN-γ and IL-4 were estimated in the peripheral blood mononuclear cells (PBMC) stimulated with Phorbol 12-myristate 13-acetate (PMA) by means of an enzyme-linked immunoassay (ELISA) kit,[29] are presented in the on-line supplement.

Determination of chemokines in the serum

Samples of venous blood (4 mL)were kept for 1 h at room temperature and then centrifuged at 3000× *g* at 4 °C for

Table 4 Cell counts and Th_1/Th_2 ratio in peripheral blood and intracellular IFN-γ and IL-4 during the DYAR and PBS.

Patients $n = 22$	Before the challenge	After the challenge (hrs)							
		1	6	12	24	36	48	56	72
Leukocytes[a]									
-DYAR	8.3 ± 0.8	9.5 ± 0.4	10.5 ± 0.9	11.0 ± 1.4	12.5 ± 0.9	**13.4 ± 0.5***	**12.9 ± 0.3***	10.3 ± 0.4	10.1 ± 0.6
-PBS	8.0 ± 0.2	7.9 ± 1.0	7.3 ± 0.1	8.6 ± 0.7	8.2 ± 0.7	8.1 ± 1.0	9.1 ± 0.5	8.2 ± 0.8	8.0 ± 0.3
Eosinophils[b]									
-DYAR	293 ± 22	334 ± 29	307 ± 21	316 ± 19	284 ± 20	269 ± 15	278 ± 30	241 ± 51	286 ± 25
-PBS	279 ± 26	288 ± 34	297 ± 28	289 ± 30	276 ± 27	268 ± 22	273 ± 20	266 ± 28	279 ± 19
Neutrophils[c]									
-DYAR	6.9 ± 0.5	6.9 ± 0.8	7.5 ± 0.4	**9.3 ± 0.8***	**9.5 ± 0.7***	**9.8 ± 0.6***	**8.5 ± 0.2***	7.0 ± 0.9	6.8 ± 0.3
-PBS	5.9 ± 1.1	6.2 ± 0.5	6.6 ± 0.7	7.1 ± 0.8	6.9 ± 0.4	7.2 ± 0.3	[illegible]	6.3 ± 0.8	6.4 ± 0.5
Thrombocytes[d]									
-DYAR	318 ± 33	351 ± 27	336 ± 30	349 ± 31	338 ± 34	427 ± 35	396 ± 25	[illegible]22 ± 29	317 ± 24
-PBS	289 ± 26	337 ± 20	328 ± 22	275 ± 17	316 ± 23	338 ± 30	336 ± 21	[illegible]95 ± 33	275 ± 15
Differential counts[e]									
=basophils									
-DYAR	0.07	0.09	0.11	0.09	0.10	[illegible]8	0.06	0.06	0.10
-PBS	0.09	0.11	0.08	0.05	0.08	[illegible]09	0.05	0.07	0.05
=eosinophils									
-DYAR	0.30	0.52	0.40	0.5	0.4	0.52	[illegible]5	0.4 1	0.28
-PBS	0.25	0.30	0.52	0.35	0.25	0.40	[illegible]25	0.30	0.25
=neutrophils									
-DYAR	6.7	6.9	7.7	7.4	**9.[illegible]**	**[illegible]***	**8.6***	**8.9***	7.3
-PBS	6.4	6.5	6.7	5.9	6.3	6.[illegible]	5.8	7.2	6.4
=monocytes									
-DYAR	0.4	0.7	1.2	**1.7***	**2.0***	**1.9***	1.0	0.8	0.5
-PBS	0.2	0.4	0.9	0.5	0.8	0.7	05	0.2	0.3
=lymphocytes									
-DYAR	3.1	3.0	2.9	3.4	**[illegible].7***	**4.8***	**4.3***	2.8	2.5
-PBS	2.5	2.2	3.4	3.5	2.[illegible]	2.7	2.5	2.6	2.7
Th_1/Th_2 ratio (%)[f]									
-DYAR	8.0 ± 1.5	8.9 ± 2.1	9.9 ± 3.0	[illegible]1 ± 4.[illegible]	**[illegible].0 ± 4.0***	**16.5 ± 3.1***	**16.2 ± 3.4***	**15.0 ± 3.5***	9.0 ± 3.3
-PBS	7.5 ± 3.2	8.0 ± 2.1	9.3 ± 2.0	7.[illegible] ± 2.4	8.3 ± 3.6	8.7 ± 2.5	9.0 ± 2.5	8.1 ± 2.3	7.7 ± 2.6
IFN-γ (pg/mL)[g]									
-DYAR	207 ± 99	190 ± 86	227 ± [illegible]05	264 ± [illegible]9	**367 ± 102***	**436 ± 107***	**400 ± 91***	**365 ± 113***	248 ± 79
-PBS	193 ± 75	211 ± 84	22[illegible] ± 10[illegible]	205 ± 9[illegible]	209 ± 103	194 ± 110	192 ± 86	195 ± 100	177 ± 88
IL-4 (pg/mL)[g]									
-DYAR	20.9 ± 2.0	23.6 ± 3.3	[illegible]1 ± 2[illegible]	30.[illegible]9	27.4 ± 3.5	26.0 ± 2.9	24.2 ± 2.8	25.7 ± 3.4	26.3 ± 3.5
-PBS	22.5 ± 4.1	26.0 ± 2.[illegible]	30.[illegible].1	28.6 ± 2.5	29.1 ± 2.4	26.5 ± 3.1	23.8 ± 3.2	25.0 ± 2.3	23.6 ± 2.9

Values = mean ± SD; Statistic[illegible]ignificance as [illegible]pared with healthy subject values: * = $p < 0.05$ (in bold), PBS = phosphate buffered saline.

[a] Normal value 4.0—10.0[illegible] 10^9/L.

[b] Normal value < 300 × 10^6/L.

[c] Normal value 2.0—[illegible]2 × 10^9/L.

[d] Normal value 150[illegible]400 × 10^9/L.

[e] Normal values [illegible]10^9/L): [illegible] = <0.2; *E* = <0.50; *N* = 2.0—7.2; *M* = 0.2—1.0; *L* = 1.0—4.0 × 10^9/L.

[f] Determined b[illegible] means o[illegible]low-cytometry after stimulation with PMA (phorbol 12-myristate 13-acetate) (means ± SEM).

[g] Determined by [illegible]ns[illegible] ELISA a[illegible]r "in vitro" stimulation of mononuclear cells (PBMC) with PMA (means ± SEM).

10 min. The se[illegible]m at[illegible]ts were stored at −70 °C until analysis. [illegible]hemokines were determined by means of commerci[illegible]nzyme-linked immunoassay (ELISA, EIA) kits (R & D Syste[illegible] Minneapolis/MN, USA), according to the manufacturers' recommendations, by a double-blind schedule. All measurements were performed in duplicate. The minimal detection limits of the ELISA kits, in *pg/mL*, were as follows: CCL 1 (7.8), CCL 2 (5.0), CCL 3 (7.0), CCL 4 (4.0), CCL 5 (5.0), CCL 7(7.8), CCL 11 (5.0), CCL 13 (7.8), CCL 17 (7.0), CCL 18 (8.0), CCL 19 (0.87), CCL 20 (0.87), CCL 22 (62.5), CCL 23 (15.0), CCL 24 (2.5), CCL 25 (62.5), CCL 26 (2.3), CCL 27 (1.6), CXCL 1 (5.0), CXCL 8 (3.5), CXCL 9 (3.8), CXCL 10 (1.7), CXCL 11 (3.0). The inter-assay coefficients of variations for these kits were less than 9%, whereas the intra-assay coefficients of variations were less than 8%.

Statistical analysis

The initial and repeated DYAR and PBS controls were analyzed by means of fitting polynomials to the mean curves over time, eight time points within 120 min and twenty-five time points up to 72 h after the allergen or PBS challenge. The hypotheses were tested by the generalized multivariate analysis of the variance model (MANOVA).[35]

The course of concentration changes of particular chemokines in blood during the DYAR and PBS controls has also been analyzed by means of the MANOVA method.[35]

The pre-challenge serum concentrations of particular chemokines measured both in the individual DYAR patients and their mean values calculated also for all patients within the same group were compared with the mean concentration values of the corresponding chemokines measured in the healthy control subjects and statistically evaluated by means of a Wilcoxon matched-pair signed rank test.

The individual post-challenge values of particular chemokines recorded at each of the time points during the repeated DYAR and PBS control in each of the patients were compared with their pre-challenge value and additionally evaluated by Wilcoxon matched-pair signed rank test. The single post-challenge chemokine values measured at each of the time points during the repeated DYAR were compared with the corresponding post-challenge PBS values and additionally evaluated by Mann—Whitney *U* test.

The mean concentration values of particular chemokines recorded singly in patients of the control groups (IAR, LAR, DLAR) were compared with the mean values of chemokines measured singly in control healthy subjects and evaluated by Wilcoxon matched-pair rank test (Table 3). A $P < 0.05$ was considered to be statistically significant value for all above mentioned tests.

Results

The isolated form of DYAR recorded in 12 patients, beginning within 26—32 h, reaching maximum within 32—48 h and resolving within 56 h after the allergen challenge (Fig. 1), was statistically highly significant both in comparison of the post-challenge with the pre-challenge FEV_1 values ($p < 0.001$) and as compared with the PBS control values ($p < 0.001$).

The repeated DYAR was also highly significant both comparing the post-challenge with the pre-challenge FEV_1 values ($p < 0.001$) and as compared with the PBS controls ($p < 0.001$) (Fig. 1). No statistically significant differences were found between the initial and the repeated DYAR ($p > 0.1$). No significant differences were found in the appearance of DYAR with respect to the individual allergens ($p > 0.1$) (Table 2). No significant differences in the post-challenge FEV_1 values, with respect to the pre-challenge FEV_1 values, were recorded during the initial as well as repeated PBS control challenges ($p > 0.05$, $p > 0.1$ respectively).

The DYAR patients demonstrated allergen-relevant positive immediate skin response in 41%($n = 9$), late skin response in 14 ($n = 3$) and delayed skin response in 45% ($n = 10$), decreased bronchial histamine threshold in 78% (Table 1) and non-increased immunoglobulin concentrations in the serum.

The DYAR was associated with increased blood leukocyte, neutrophil and lymphocyte counts, significant changes in the Th_1/Th_2 cell ratio in peripheral blood in favor of Th_1 cells ($p < 0.01$), significant increase in the intracellular concentration of IFN-γ ($p < 0.05$), but not of IL-4 ($p > 0.05$) (Table 4).

In DYAR patients, the pre-challenge serum concentrations of CCL 20, CXCL 1, CXCL 9 and CXCL 11 were slightly elevated, whereas concentration of CCL 25 was slightly decreased, as compared with the healthy control subjects. No statistically significant differences have been found in the pre-challenge concentrations of other chemokines between the DYAR patients and the healthy control subjects ($p > 0.05$) (Table 3).

The DYAR was associated with significant changes ($p < 0.05$) in the serum concentrations, of the following chemokines (Table 3): (a)an increased serum concentration of CCL 2 at 6, 12 and 24 h (followed by its decrease at 48 h), CCL 3 at 6, 12, 24 and 36 h, CCL 4 at 6, 12, 24, 36 and 48 h, CCL 7 at 12, 24 and 36 h, CCL 20 at 1 and 6 h, CXCL 1 at 1 and 6 h, CXCL 8 at 1, 24 and 36 h, CXCL 9 at 1, 6, 12 and 24 h (followed by its decrease at 48 and 56 h), CXCL 10 at 24, 36 and 48 h, and CXCL 11 at 1, 6 and 12 h; (b) a decreased serum concentration of CCL 5 at 6, 12 and 24 h, CCL 16 at 6, 12, 24 and 36 h, CCL 17 at 6, 12 and 24 h, CCL 22 at 6, 12 and 24 h, CCL 24 at 1, 6 and 12 h, and CCL 26 at 6 and 12 h, as compared both with their pre-challenge values and with the PBS control values. No significant changes in the serum concentrations of chemokines were measured during PBS control challenge ($p > 0.1$) (Table 5).

Control subjects

The pre-challenge serum concentrations of some chemokines measured in the control patients with IAR, LAR and DLAR differed from those recorded in the healthy control subjects (Table 3).

Discussion

The DYAR, an unique asthmatic response phenotype, recorded in approximately 12% patients with allergic bronchial asthma,[29] differs from the other types of asthmatic response, such as IAR and LAR, not only with respect to its clinical features including the clinical course, but also in its association with other "in vivo" and "in vitro" diagnostic parameters, in the immunologic features and in association with changes of various factors in blood (Table 1).[12,16—32,36—39]

Results of our previous studies demonstrated association of DYAR with significantly increased plasma concentrations of LTB_4 and MPO,[31] adhesion molecules, especially sICAM-1, sVCAM-1, sPECAM-1, sE- and L-selectin,[30] and of some cytokines, such as IFN-γ, IL-2, IL-18, G-CSF, TNF-α, TGF-β, decreased plasma concentrations of IL-7 and IL-12p70. The concentrations of the other cytokines, especially IL-1β, IL-4, IL-5, IL-6, IL-8, IL-12p40 and GM-CSF did not change significantly.[32]

The DYAR was also accompanied by changes in the Th_1/Th_2 ratio, in favor of Th_1 cells,[29—32] and increased intracellular concentrations of IFNγ and IL-2, but not IL-4 or IL-

Table 5 Concentrations of particular chemokines in *pg/mL* (mean ± SEM) recorded during DYARs and PBS control tests.

Patients $N = 22$	Before the cha[illegible]ge	After the challenge (hrs)							
		1	6	12	24	36	48	56	72
CCL 1 (I-309)									
-DYAR	<7.8	<7.8	<7.8	<7.8	8.5 ± 0.4	8.2 ± 0.3	<7.8	<7.8	<7.8
-PBS	<7.8	[illegible]	<7.8	<7.8	<7.8	<7.8	<7.8	<7.8	<7.8
CCL 2 (M[illegible]-1)									
-DYAR	30.2[illegible] 1.6	28.9 ± 2.1	**57.5 ± 3.9***	**84.1 ± 2.7***	**93.0 ± 4.5***	11.3 ± 1.4	**< 5.0***	9.2 ± 0.8	27.0 ± 1.0
-PBS	33[illegible] ± 0.4	[illegible].0 ± 1.3	35.2 ± 1.7	32.3 ± 2.2	34.7 ± 3.1	30.9 ± 2.1	33.05 ± 1.8	28.4 ± 0.9	31.2 ± 1.7
CCL 3 (MIP-1α)									
-DYAR	8.5 ± 0.3	9.8 ± 1.0	**28[illegible] ± 2.1***	**41.7 ± 2.9***	**48.3 ± 3.4***	**27.9 ± 1.3***	12.4 ± 0.7	<7.0	8.4 ± 0.5
-PBS	8.2 ± 0.5	8.7 ± 0.6	9.5 ± [illegible]	9.2 ± 0.4	9.8 ± 1.1	8.6 ± 0.9	9.3 ± 1.0	10.2 ± 1.3	8.0 ± 0.6
CCL 4 (MIP-1β)									
-DYAR	6.6 ± 0.2	5.9 ± 0[illegible]	7.4[illegible] 2.8	**29.0 ± 3.1***	**25.5 ± 2.0***	**19.4 ± 2.2***	**18.7 ± 1.3***	9.0 ± 0.2	6.8 ± 0.4
-PBS	7.0 ± 1.0	7.2 ± 0.6	8[illegible] [illegible].1	5.9 ± 0.3	5.7 ± 0.6	6.6 ± 1.0	7.4 ± 0.9	7.1 ± 0.8	7.1 ± 2.3
CCL 5 (RANTES)									
-DYAR	15.7 ± 2.3	17.9 ± 1.5	**< 5.0***	**< 5[illegible]**	**< 5.0***	7.8 ± 1.3	14.9 ± 2.0	16.5 ± 1.7	16.1 ± 0.9
-PBS	13.9 ± 1.2	15.3 ± 0.8	14.6 ± [illegible].0	[illegible].1 ± [illegible].7	15.0 ± 1.4	15.5 ± 0.7	13.6 ± 1.1	14.4 ± 1.3	14.9 ± 1.2
CCL 7 (MCP-3)									
-DYAR	9.4 ± 0.3	10.1 ± 1.0	10.7 ± 1.8	**[illegible].5 [illegible].6***	**[illegible] ± 0.3***	**35.9 ± 1.0***	12.1 ± 0.7	11.0 ± 2.1	10.2 ± 1.3
-PBS	9.6 ± 0.8	11.0 ± 0.5	10.2 ± 0.4	9.1[illegible].2	8.5 ± [illegible].7	10.8 ± 0.6	10.0 ± 0.9	10.3 ± 1.0	9.0 ± 0.8
CCL 11 (Eotaxin-1)									
-DYAR	7.7 ± 2.1	7.2 ± 1.0	**< 5.0***	**< 5[illegible]***	**< 5.0***	**< 5.0***	5.3 ± 0.2	6.8 ± 1.0	7.1 ± 2.0
-PBS	7.8 ± 1.2	8.0 ± 1.7	8.1 ± 1.0	7.2 ± 0.4	7.9 ± 0.6	[illegible] ± 1.5	7.5 ± 1.7	7.2 ± 0.5	8.0 ± 1.0
CCL 13 (MCP-4)									
-DYAR	112.3 ± 4.1	105.1 ± 4.6	92.0 ± 3.2	98.4 ± 2.9	107.8 ± 3.5	95.0 ± [illegible]	109.3 ± 5.1	88.5 ± 3.4	96.9 ± 3.0
-PBS	89.8 ± 3.2	93.9 ± 2.8	106.5 ± 1.7	101.3 ± 2.4	96.1 ± 1[illegible]	103.[illegible] 2.2	90.9 ± 1.7	93.5 ± 2.0	91.4 ± 0.8
CCL 17 (TARC)									
-DYAR	68.3 ± 3.5	54.9 ± 4.1	**< 7.0***	**< 7.0***	**< 7.0***	[illegible].9	56[illegible] ± 4.2	65.5 ± 3.8	63.0 ± 2.7
-PBS	71.5 ± 5.6	61.8 ± 4.9	58.4 ± 3.3	61.6 ± 2.5	70.2 ± 1.9	[illegible].4 ± 1.[illegible]	[illegible].0 ± [illegible].7	62.0 ± 2.3	67.5 ± 1.6
CCL 18 (PARC)									
-DYAR	31.6 ± 4.8	38.0 ± 4.2	29.7 ± 3.0	25.9 ± 2.2	36.4 ± 2.8	30.[illegible] [illegible].7	28.9 ± 1.6	32.0 ± 2.9	34.2 ± 1.5
-PBS	28.8 ± 3.0	30.5 ± 2.6	31.3 ± 2.1	29.7 ± 1.5	25.3 ± 1.0	34.8 ± [illegible].0	30.3 ± 2.[illegible]	26.6 ± 1.7	30.9 ± 2.0
CCL 19 (MIP-3β)									
-DYAR	12.7 ± 1.5	14.7 ± 3.4	11.2 ± 2.5	13.8 ± 3.9	12.2 ± 1.3	12.9 ± 2.2	10.8 ± [illegible].5	11.3 ± 1.9	11.7 ± 1.4
-PBS	10.9 ± 0.8	10.5 ± 1.7	12.7 ± 3.1	11.3 ± 1.8	10.8 ± 2.2	11.0 ± 1.4	11.5 ± 0.7	10.8 ± 3.0	11.2 ± 1.1
CCL 20 (MIP-3α)									
-DYAR	19.5 ± 0.6	**38.7 ± 3.5***	**46.9 ± 2.7***	22.1 ± 1.3	20.8 ± 2.0	23.9 ± 2.5	18.4 ± 1.0	17.2 ± 2.3	18.8 ± 1.6
-PBS	21.4 ± 3.0	25.5 ± 2.9	22.0 ± 1.1	23.8 ± 2.4	24.5 ± 1.3	25.6 ± 1.9	20.0 ± 1.7	21.2 ± 1.5	20.9 ± 2.0
CCL 22 (MDC)									
-DYAR	237.2 ± 21	225.3 ± 18	**73.8 ± 12***	**< 62.5***	**85.9 ± 31***	219.4 ± 26	208.5 ± 31	229.2 ± 25	216.8 ± 15
-PBS	211.0 ± 14	203.1 ± 15	234.6 ± 24	223.8 ± 29	235.2 ± 20	216.9 ± 18	242.0 ± 27	236.5 ± 17	228.5 ± 23
CCL 23 (MIP-3)									
-DYAR	83.4 ± 6.9	75.2 ± 5.4	91.7 ± 4.9	86.5 ± 3.8	79.0 ± 6.1	82.0 ± 3.9	90.0 ± 4.4	66.7 ± 5.2	77.9 ± 3.8

-PBS	70.[illegible]	[illegible] ± 2.9	80.3 ± 3.4	75.8 ± 2.5	84.0 ± 4.3	73.7 ± 2.5	84.2 ± 3.1	73.0 ± 3.9	71.8 ± 2.3
CCL 24 ([illegible]taxin-2)									
-DYAR	135.[illegible] ± 11	**51.7 ± 4.8***	**43.2 ± 3.9***	**47.4 ± 5.3***	105.2 ± 7.2	128.5 ± 4.9	133.0 ± 3.6	127.5 ± 2.8	131.0 ± 4.2
-PBS	144 ± 8.[illegible]	[illegible]1.0 ± 6.5	133.9 ± 5.1	140.0 ± 6.1	132.7 ± 5.5	125.0 ± 7.3	129.5 ± 4.0	138.0 ± 3.5	142.3 ± 2.7
CCL 25 (TECK)									
-DYAR	<62.5	<62.5	<[illegible]5	<62.5	<62.5	<62.5	76.1 ± 3.6	75.8 ± 4.0	<62.5
-PBS	<62.5	<62.5	<62.5	<62.5	<62.5	<62.5	<62.5	<62.5	<62.5
CCL 26 (Eotaxin-3)									
-DYAR	182.0 ± 12	104.5 ± [illegible]	**71[illegible] ± 6.8***	**80.3 ± 5.9***	156.5 ± 8.0	167.3 ± 10	177.0 ± 12	169.5 ± 8.8	175.4 ± 9.3
-PBS	165.3 ± 9.1	155.0 ± [illegible].7	1[illegible] ± 11	174.6 ± 15	169.5 ± 9.4	181.2 ± 13	156.4 ± 9.9	158.0 ± 12	172.3 ± 8.0
CCL 27 (CTACK)									
-DYAR	361.5 ± 34	320.1 ± 28	316.4 ± [illegible]	353.[illegible]6	347.9 ± 31	330.8 ± 25	329.6 ± 18	355.0 ± 25	344.6 ± 14
-PBS	327.0 ± 13	345.5 ± 23	302.7 ± [illegible]0	[illegible].6 ± [illegible]3	329.5 ± 11	348.0 ± 14	335.9 ± 31	341.0 ± 22	353.3 ± 9.2
CXCL 1 (GRO-α)									
-DYAR	65.0 ± 7.2	**189.4 ± 6.3***	**214.5 ± 8[illegible]**	[illegible]2.7[illegible] 5.4	[illegible] ± 4.8	51.3 ± 2.7	55.0 ± 1.9	59.4 ± 1.5	61.2 ± 2.4
-PBS	63.8 ± 5.1	59.0 ± 4.8	52.9 ± 2.5	61.[illegible]4.9	58.3[illegible]3.5	60.0 ± 1.9	53.3 ± 2.5	62.5 ± 2.1	67.0 ± 3.5
CXCL 8 (IL-8)									
-DYAR	8.1 ± 1.3	7.4 ± 2.0	14.5 ± 3.1	**29.[illegible] ± 4.[illegible]**	**25.9 ± 3.6***	**21.0 ± 2.8***	10.4 ± 1.0	**< 3.5***	7.3 ± 0.9
-PBS	7.7 ± 0.6	8.9 ± 3.2	6.8 ± 1.5	7.2 ± 0.5[illegible]	8.3 ± 2.4	[illegible].5 ± 3.0	8.2 ± 0.9	7.5 ± 0.6	7.8 ± 1.3
CXCL 9 (MIG)									
-DYAR	22.4 ± 0.5	**83.6 ± 3.7***	**98.0 ± 4.9***	**75.8 ± 5.1***	**[illegible]9.6 ± 3.5***	34.0 ± [illegible]	**7.5 ± 2.9***	**6.1 ± 1.8***	18.4 ± 0.8
-PBS	20.8 ± 1.0	25.5 ± 2.4	30.0 ± 2.2	23.6 ± 1.5	27.0 ± 2.[illegible]	22.1 [illegible].9	24.7 ± 1.3	25.0 ± 1.4	21.5 ± 1.6
CXCL 10 (IP-10)									
-DYAR	15.4 ± 2.3	13.8 ± 1.9	12.6 ± 1.2	18.7 ± 2.5	**39.4 ± [illegible].1***	**4[illegible] ± [illegible].8***	**37.[illegible] ± 1.5***	19.4 ± 2.2	17.1 ± 1.3
-PBS	15.9 ± 1.5	11.6.±0.7	14.3 ± 2.1	10.5 ± 0.8	13.7 ± 1.1	[illegible].6 ± [illegible]	[illegible] [illegible].4	16.3 ± 1.5	16.1 ± 2.0
CXCL 11 (I-TAC)									
-DYAR	51.2 ± 4.3	**96.5 ± 4.2***	**109.8 ± 5.4***	**119.0 ± 3.7***	66.8 ± 3.5	59[illegible] ± 3.4	63.0 ± 2.9	55.1 ± 3.3	56.7 ± 2.8
-PBS	49.0 ± 2.2	52.3 ± 1.7	54.9 ± 3.5	58.8 ± 1.4	54.0 ± 2.3	46.7 ± [illegible].5	48.2 ± 3.[illegible]	50.5 ± 2.8	48.1 ± 1.3

Values = mean ± SEM; Statistical significance as compared with the pre-challenge (baseline) values: * = $p < 0.05$ (in bold); P[illegible] = phospha[illegible]buffered saline.

5, in blood lymphocytes.[29] The significantly increased plasma concentrations of TGF-β and a slightly increased serum concentration of IL-10, might be suggestive for a possible involvement of the Th_3 —cells in the mechanism underlying the DYAR.[32] A possible role of another important T-helper cell sub-set, the Th 17 cells producing IL-17 A-F, in the mechanism(s) leading to the DYAR, has not yet been investigated by us for technical reasons. The increased counts of total leukocytes, neutrophils and monocytes, but not those of eosinophils, accompanying the DYAR,[29–32] is partly consistent with the other investigators' findings of a separate entity, the "non-eosihophilic/neutrophilic" asthma phenotype.[40–50].

Chemokines represent a very important part of immunologic mechanisms and processes.[6–9,11–15,19–22,28,38,39,51–58] They act predominantly as chemoattractants and signal transmitters among various cell types, but they also display a number of other effects, such as stimulation/activation *vs* inhibition effects on various intra- and extracellular processes and cellular traffic, affecting the maturation and differentiation period of various cell lineages.[6–9,11,13,38,54–57] They operate in a close relation with other factors participating in the immunologic mechanisms, such as classical mediators, cytokines, cell surface molecules (CD), adhesion molecules and cellular constituents released by various cell types.[2,3,5–9,11,13,15,20,38,39,51–57] Most of their effects are realized through the interaction with their ligands, which are specific receptors expressed on the target cells.[5–9,11–15,19,20,39,51,52,54,56,57] The role of chemokines in allergic bronchial asthma has been studied from various points of view.[2,5–8,11,12,14,15,19–22,36,38,51,55] In most of these studies the chemokines were determined by means of a single measurement in bronchial asthma patients.[2,14,15,51,53] In some of these studies the chemokines were measured in bronchial lavage (BAL) fluid, sputum, bronchial biopsies following. the segmental/intrabronchial challenge with allergen[5,12,20–22,38] The papers dealing with the measurement of the chemokine concentration changes in blood during the individual types of asthmatic response to allergen challenge are not numerous.[12] Moreover, in some studies the chemokine concentrations were measured after "in vitro" stimulation of the cells isolated from peripheral blood, induced sputum or BAL fluid.[38]

A single determination of the concentrations of immunologic factors, such as chemokines, in the peripheral blood, sputum or BAL fluid in asthmatics related only to a certain time point and not to a specific and controlled event, such as provocation test with allergen, does not generate information on the dynamic course of the chemokine changes during the whole immunologic process and clinical response. This is in only be achieved by measurements of these factors before and repeatedly after the bronchial segmental provocation tests with allergen, techniques by which the concentration changes of particular factors, e.g. chemokines, in blood or in BAL fluid can be related to a certain type of asthmatic response.[5,12,20–22,38]

The bronchial challenge techniques have a number of advantages and disadvantages. The advantage of the segmental challenge is the provocation of a limited lung area with a small allergen amount and direct access to the immunologic reaction site. Its disadvantage is an exclusion of the large part of the respiratory system (from the mouth to the secondary bronchi) which may regularly be the site of the initial stages of the immunologic process leading to the asthmatic response. This method is relatively laborious, requires application of an anesthetic agent and the less natural BAL technique can be a burden for the patient. The advantage of the inhalation technique is its simulation of the natural allergen exposure upon including of all parts of the airways, relatively low strain for the patient and low risk of post-intervention complications besides the expected bronchoconstriction. Its disadvantage is the need of a rather large effective allergen dose.[16,17,29–32,59,60]

Generally, there is a dearth of data concerning the appearance and course changes of chemokines in the peripheral blood during the particular types of asthmatic response due to the inhalational bronchial provocation with allergen (BPT). Moreover, there is also a scarcity of comprehensive information concerning the chemokine profiles in peripheral blood, sputum and BAL fluid, in healthy subjects which could be used as reference values.[5]

The changes in chemokine concentrations during the DYAR due to bronchial challenge with allergen in patients with allergic bronchial asthma have not yet been investigated. Our current study, being probably the first one on this topic, presents a number of interesting and unique data. These results concerning the chemokine profile associated with the DYAR differ distinctly from the chemokine profiles found by other investigators in patients developing either the IAR or the LAR.[5,12,20–22,38,55] The different chemokine profiles would be suggestive for involvement of different immunologic mechanisms in the DYAR on one hand and in the IAR and LAR on the other hand.

One of the remarkable clinical features recorded in patients developing the DYAR was appearance of various types of skin response, such as immediate late and delayed, to the same allergens as those causing the positive DYAR (Table 1). Unfortunately, we have not yet a satisfactory explanation for this variation in skin tests. Perhaps, it may be related to some differences in the immunologic system of the skin and that of the airways, and to different processing of the allergens on these different locations.

Another interesting finding was the non-increased serum concentrations of the immunoglobulins, especially of the allergen-specific IgE, in the DYAR patients, whereas in asthmatics of the 3 control groups (IAR, LAR, DLAR) the positive allergen-specific IgE antibodies were found. Regarding these findings, similar to our previous results,[29,30,32] the involvement of the IgE-mediated hypersensitivity in the mechanism(s)underlying the DYAR seems to be unlikely.

The pre-challenge serum values of some chemokines recorded in the DYAR patients differed slightly from those measured in control healthy subjects (Table 3). The pre-challenge serum values of some chemokines measured in the control patients exhibiting IAR, LAR or DLAR were also slightly increased as compared with the values recorded in the healthy control subjects (Table 3). These findings are partly consistent with other investigators' data of increased levels of CCL 11, CCL 13, CCL 20, CCL 22, CCL 24, CCL 26, CXCL 8 in the serum[2,12,61–63] or CCL 11, CCL 22, CCL 24, CCL 26, CXCL 8 in the BAL fluid of asthmatics.[5,12,21,53,62]

In contrast, the post-challenge chemokine profiles recorded in the serum during the DYAR were characterized by the significantly increased concentrations of CCL 2, CCL 3, CCL 4, CCL 7, CCL 20, CXCL 1, CXCL 8, CXCL 9, CXCL 10 and CXCL 11, significantly decreased concentrations of CCL 5, CCL 11, CCL 17, CCL 22, CCL 24 and CCL 26, and non-altered levels of CCL 1, CCL 13, CCL 18, CCL 19, CCL 23, CCL 25 and CCL 27.

These results, together with significantly increased counts of total leukocytes, neutrophils, lymphocytes and monocytes, but not of eosinophils, absence of allergen-specific IgE antibodies in the serum, unchanged concentrations of other immunoglobulins, changes of Th_1/Th_2 ratio in favor of Th_1 —cells, and increased concentration of intracellular IFN-γ but not that of IL-4, in PBMC, together with our previous findings[29—32], would suggest possible involvement of the cell-mediated hypersensitivity, upon participation of Th_1 lymphocytes, neutrophils, monocytes, epithelial and endothelial cells, and probably also NK, macrophages and dendritic cells, in the mechanisms underlying the DYAR.

Unfortunately, no reliable data concerning the other T cell subsets, such as Th 17 and Treg and their products as well as the activation degree and other features of the particular chemokine membrane receptors on the various cell types in relation to the DYAR, are available. Moreover, the cytologic and immunologic features in BAL fluid during the DYAR have not yet been studied.[29—32] These facts may be regarded as a certain deficit of the current study and they have to be certainly investigated in the following studies.

It can be summarized that, the DYAR, a relatively unique asthmatic response phenotype, displays a number of remarkable clinical and immunologic features,[29—32] such as (a) clinical manifestation within 26—56 h after the allergen exposure, (b) clinical symptoms represented predominantly by pronounced dyspnea; (c) unsatisfactory correlation with skin prick tests and bronchial histamine threshold, (d) absence of allergen-specific IgE antibodies in the serum and of blood eosinophilia; (e) increased counts of leukocytes, neutrophils, monocytes and lymphocytes in peripheral blood; (f) shifting of the Th_1/Th_2 ratio in peripheral blood in favor of the Th_1 cells; (g) increased blood concentrations of IFN-γ, IL-2, IL-18, G-CSF, TNF-α, TGF-β, but not IL-4 or IL-5; (h) increased blood concentrations of CCL-3,-4,-20, and CXCL-1,-8,-9,-10 and -11. These facts may indicate a possible involvement of the cell-mediated hypersensitivity mechanism in the DYAR. Regarding these facts, it would be very difficult to predict the possible existence of DYAR from the standard diagnostic procedures performed in the patients with bronchial asthma and this response type can therefore be overlooked in the practice. The bronchial challenge with allergen seems to be only reliable procedure for detection and definite confirmation of the DYAR.

In conclusion, the results of the current study, together with our previously published results,[29—32] suggest a possible involvement of the cell-mediated hypersensitivity mechanisms in the DYAR. Nevertheless, further concurrent clinical and immunologic research, including BAL, airway biopsy, other chemokines, cytokines, chemokine receptors on the membrane of the circulating as well as in the bronchial tissue residing cell types, other T-cell subsets, such as Treg, Th 17, and various transcription factors, will be needed to clarify exactly the immunologic mechanism(s) underlying this clinical phenomenon.

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Conflict of interest

Author has no conflict of interest to be disclosed.

Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.rmed.2012.09.013.

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